THE MOSAIC DEVELOPMENT OF THE ASCIDIAN EGG

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The development of ascidian eggs has long been considered, as the result of the classical investigations by Conklin, to be the perfect example of the mosaic rather than the regulative type.

Different blastomeres of dividing eggs were killed or sufficiently injured to inhibit further development and the development of the remaining blastomere or blastomeres was followed. He found that each blastomere developed only into the tissues and organs that it would have produced had it remained a part of intact egg or embryo. In no case did a whole but dwarf larva result from one of the first two or four cells. The left blastomere of the 2-cell stage gave rise to the organs of the left side only. The anterior two blastomeres of the 4-cell stage gave rise to epidermal, neural, and chordal tissues, but failed either to gastrulate or to form a tail.

In general the organization of amphibian eggs is similar to that of ascidian eggs, formative substances appearing rather later, although Hall has recently shown that they are delimited as early as the 8-cell stage. Isolated blastomeres of the 2-cell stage, however, may give rise either to a whole larva of half the normal size or to a right or left half-larva of normal size, according to whether or not there has been reorganization of the cell contents.

In consequence of this it has seemed possible that the absence of regulation or reorganization in Conklin's experiments may have been due to the fact that the injured blastomeres were left in situ within the egg membrane. The presence of their inert mass in contact with the surviving part may have had an inhibitory effect. This possibility has been emphasized by Reverberi's conclusions, based upon experiments with *Ciona* eggs, that the eggs of ascidians should be placed properly within the regulative class.

The great obstacle to experimental work with ascidian eggs has been the difficulty of removing the egg membranes and follicle cells without injuring the ovum. It is partly the object of this paper to record a method by which this may be done. Several ascidian eggs are shown in Fig. 1, and it is seen that there is considerable variation in the character of these membranes.

It has already been shown (Berrill, 1929) that the membranes are normally digested from within by a proteolytic enzyme secreted by the developing tadpole, that the enzyme is fairly stable and is active within the limits pH 7.0–10.0. Trypsin digests the membranes, but only very slowly, so that the protease concerned is probably a tryptic ferment of a less specialized nature than trypsin itself.

There are, accordingly, two means by which membranes of ascidian eggs may be removed. The enzyme produced by developing ascidian tadpoles may be collected and concentrated, or a suitable and more readily obtainable substitute may be found. It was discovered that *Ciona*, *Phallusia*, *Ascidia*, and *Ascidiella* eggs remained about 100 per cent viable at 18° C. for a little over twenty-four hours, so that any enzyme mixture to be of use must be effective within that period.

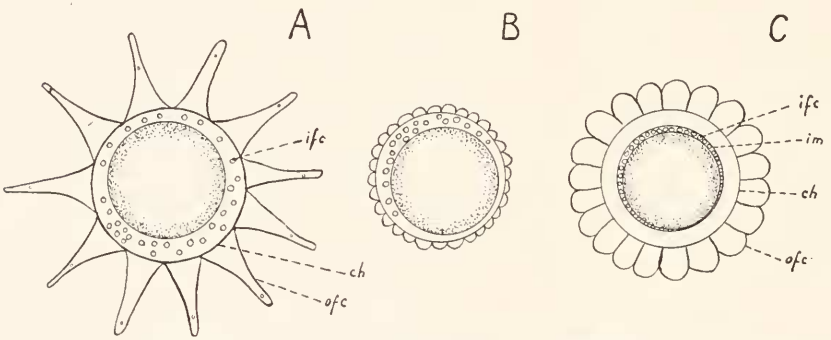

FIG. 1. Ascidian eggs. *A*, *Ciona intestinalis; B*, *Molgula* or *Polycarpa; C*, *Ascidiella aspersa*. *ifc*, inner follicle cell; *im*, inner membrane; *ch*, chorion or egg-membrane; *ofc*, outer follicle cell.

The natural ascidian enzyme in sufficient concentration is preferable to any substitute, but it can be obtained only from species of the family Ascidiidae (i.e., the genera *Ascidia*, *Ascidiella*, *Phallusia*) and *Ciona*. To obtain it in effective concentration it is necessary to make mass cultures of some thousands of embryos. Normal development of large numbers of eggs necessitates the use of a relatively large volume of water, the larger the better, for it is only when development has proceeded to within two or three hours of hatching that concentration becomes effective. Then the embryos are allowed to settle on the bottom of the vessels and as much supernatant water as possible is poured off. The remainder, together with the embryos, is poured into a test tube or cylinder, the embryos again allowed to settle, and the water reduced to about 5 cc. This with the embryos is then spread over the bottom of a Petri dish to ensure a supply of oxygen to the embryos, left there until hatching is complete, and the water then

collected. It should contain enough enzyme to digest off the membranes from unfertilized *Ciona* or *Ascidia* eggs in from five to ten hours. Such eggs may subsequently be fertilized.

If it is desired to remove the membranes from merely a small number of eggs, the eggs may be placed among the developing tadpoles about three hours before they are due to hatch, when their membranes will dissolve about an hour after hatching (the developing tadpoles should be close enough together to be almost touching and to form a sheet one layer deep). It may be desirable to remove the layer of outer follicle cells by washing the eggs in a bag of fine bolting silk, when the enzyme can more readily attack the underlying membrane.

Large *Ascidia* or *Ciona* are not, however, always obtainable, and it is often more convenient to use a substitute for the ascidian enzyme. Such substitutes may be obtained from any large carnivorous or omnivorous invertebrate, the most active mixtures being the stomach juices themselves rather than gland extracts or preparations.

Those actually used were the clear juices found in the stomach of decapod crustacea such as *Munida*, *Maia*, and *Homarus*. No doubt other forms would be equally effective.

Undiluted, such juices are too potent and toxic, but a dilution of one part juice with fifty or one hundred parts of sea-water is both safe and efficient. The time taken for the membranes to be removed depends not only upon the activity of the enzyme but also on the thickness of the membrane and on the temperature. At 18° C. the membranes of *Ciona*, *Molgula*, or *Ascidia* are removed in from two to four hours, but the thicker membranes of *Polycarpa* or *Styela* require more than twenty-four hours. Unfertilized eggs of *Ciona*, *Molgula*, and *Ascidia* that have had their membranes removed in this way may subsequently be fertilized and undergo normal development.

If fertilized eggs are subjected to such a mixture or if eggs are fertilized while yet within it, a somewhat unexpected feature appears. Nuclear division proceeds normally, at least to the 32-cell stage, but cytoplasmic division is inhibited. In the case of later embryos, the surface cells round off and tend to fall away. These phenomena are apparently due to surface action of the lipochrome contained in the original digestion mixture, and in consequence this method of removing the membranes is satisfactory only in the case of unfertilized eggs. In this last case the capacity for fertilization and normal development is unaffected.

Once membranes have been removed, experiments of two kinds become possible. The naked eggs may be cut with glass needles, as

used by Horstädius (1928), while blastomeres may be separated from one another. Attempts to cut the eggs were made only in the cases of *Ascidia* and *Ciona*. Those of *Ascidia* (*Ascidiella aspersa*) possessed a tough surface and a very fluid endoplasm, so that all attempts at cutting resulted in bursting them. The eggs of *Ciona*, on the other hand, are readily cut but possess such a viscous endoplasm that the injured surface does not re-form. In neither case, therefore, is experimental work of this nature feasible. It is just possible, however, that other eggs, such as those of *Molgula* or of other species of *Ascidia*, may be more suitable and of a type intermediate between those of *Ciona* and *Ascidiella*.

Separation of blastomeres, on the other hand, is very easy to effect. They may be separated with a glass needle, as used by Horstädius, or more simply merely by slight shaking. Pouring water in which there may be some hundreds of naked eggs in the 2-cell or in the 4-cell stage from one vessel to another several times results usually in the complete separation from one another of all blastomeres. There is a tendency among the blastomeres of *Ciona* eggs to fall apart in any case, though eggs and early embryos of *Ascidiella* tend to fuse on contact.

In the subsequent development of blastomeres isolated in the above manner no indication was found of any reorganization. Blastomeres isolated in the 2-cell stage invariably give rise to lateral half-embryos that gastrulate imperfectly and have but twenty instead of forty notochordal cells which, moreover, fail to interdigitate. Anterior blastomeres isolated at the 4-cell stage formed small spheres of ectoderm with about ten notochord cells loosely connected with the outer surface. Posterior blastomeres derived from the same stage formed small spheres of endoderm and mesoderm enclosed within ectodermal cells. Illustrations of such partial development are shown in Fig. 2. Each isolated blastomere apparently divides to form just those cells and tissues it would have formed had it remained a part of a whole embryo. Never was there any sign of development to form a whole larva of half or quarter normal size.

It is concluded, therefore, that Conklin's results are in no way invalidated by his failure to remove the dead or injured blastomeres or the egg membrane, and his conclusions concerning the mosaic nature of the ascidian egg are confirmed.

Reverberi, on the other hand, has reached conclusions of a very different nature. By an ingenious method of puncturing the chorion and compressing the ovum he succeeded in dividing *Ciona* eggs into two unequal parts, both parts subsequently being fertilized. His main conclusion, that the ascidian egg should definitely be assigned

to the regulative class, is based upon two discoveries. The segmentation of the small extruded part of the ovum not only commences in a plane at various angles to the first cleavage plane of the ovum proper, but proceeds in a fairly typical manner; while eggs part of which have been extruded are often able to develop to form an apparently perfect tadpole larva.

These statements are not questioned, but they are by no means incompatible with the mosaic conception.

The development of the small extruded part is normal only in so far as the first three cleavages are in the three planes of space as in the normal egg. Gastrulation and coördinated development do not occur. Since, with the exception of the Nemathelminthes, the seg-

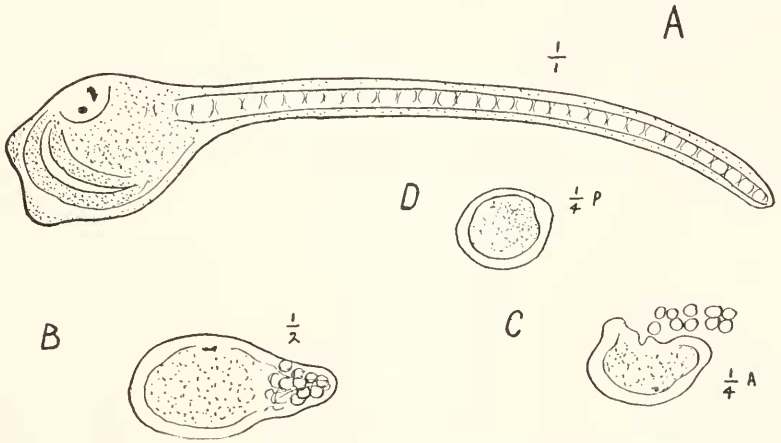

FIG. 2. Development of *Ascidiella aspersa* (24 hours at 17° C.). *A*, normal tadpole; *B*, development of isolated blastomere of 2-cell stage; *C*, of anterior blastomere of 4-cell stage; *D*, of posterior blastomere of 4-cell stage.

mentation of almost all animal eggs is uniform, the first cleavage being vertical, the second vertical at right angles to the first, and the third horizontal, the ability of part of an ascidian egg to follow this same course is alone hardly evidence of strict regulation and any other sequence would be startling. The outstanding exception to such a course, namely the segmentation of the egg of *Ascaris*, results in a very unstable configuration. The spindle tends to lie along the longest cytoplasmic axis, and the fact that a fertilized part of an ascidian egg obeys this rule of Hertwig in the first stages merely emphasizes its validity. It has no real bearing upon the mosaic or regulative nature of the egg.

The other experiments supposedly showing a regulative capacity on the part of the egg concerned the development of diploid eggs,

part of which had been extruded after fertilization. These developed to form apparently normal tadpoles of reduced size. In no case was it found possible to orientate the eggs with regard to their oöplasmic contents. The only illustrations, however, show two larvæ with tails about four-fifths full length and with a trunk region from about four-fifths normal to full size. The one with the smaller trunk has the longer tail, and if the part extruded consisted of part of the endodermal region of the ovum, the peculiarity of this tadpole is accounted for. Similarly, the large-bodied, short-tailed larva is explained if the part extruded contained more of the chordal crescent and less of the endodermal oöplasmic region. No growth in size, other than the swelling of notochord cells, occurs in *Ciona* until after the tadpole stage has been passed, so that the size of the tadpole is a fairly accurate indication of the size of the egg, and it is evident in these two cases that only a very small part can have been extruded. In any case the peculiarities of the two examples illustrated can be as readily explained on the basis of the mosaic conception as on the assumption that the egg is more regulative than is generally believed.

Summary

A new method of removing the membranes from ascidian eggs in the mass is described. Essentially the method is the use of proteolytic enzymes.

The development of isolated blastomeres is described, confirming Conklin's results that the development of each is strictly partial and that the whole development is a mosaic. It is also shown that the presence of the egg membrane and of injured or dead blastomeres in his experiments in no way invalidated those results.

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